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# A *Pseudomonas Protegens* with High Antifungal Activity Protects Apple Fruits Against *Botrytis Cinerea* Gray Mold

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## ABSTRACT

Using beneficial bacteria to control plant pathogens has become an ecofriendly alternative to the excessive use of chemicals. In this investigation, a bacterial strain RhiNA, isolated from an agricultural land in northern Algeria (Bejaia), was selected based on its ability to produce antifungal and plant growth promoting (PGP)-metabolites. It was then molecularly identified and screened for its antagonistic activity against *B. cinerea*, *Mucor* sp., *A. niger* and *A. flavus*. The isolate was also tested for its ability to attenuate gray mold caused by *B. cinerea* on apple fruits. This strain, identified as *Pseudomonas protegens*, produced several hydrolytic enzymes, IAA, siderophores, HCN, ammonia and showed potential inhibition of mycelial growth and spore fungal germination (PGI : 66,66,62 and 58% ; SGP : 14.32, 55.10, 28.92 and 10.15% against *A. niger*, *Mucor* sp., *B. cinerea* and *A. flavus*, respectively). Only 172.823 $mm^2$ of each inoculated area on apple fruits were touched by *B. cinerea*-gray mold, compared to 529.74 $mm^2$ of rotted zone in absence of the strain. *P. protegens*-RhiNA shows high efficiency against a virulent fungal strain of *B. cinerea*. Thus, it could be used as a biocontrol agent for a sustainable agriculture in future.

**Keywords***:* PGPR, Biocontrol, Phytopathogens, IAA, Siderophores, Phosphate Solubilization.

## I. INTRODUCTION

The alarming increase of human population, that is supposed to cross 9 billion by the end of 2050, requires more food and feed production; especially in countries were resources are damaged due to uncontrolled human activities and environmental degradation. Agriculture is the main source of food for humans. Its total production needs to jump by 70 to 100% to satisfy global increased demands in the next few years [1]. However, agricultural practices are threatened by various abiotic and biotic stresses, which require more resources exploitation to avoid decreased productivity [2]. Among these challenges, phytopathogens are responsible of 10 and 20% of yield losses in both developed and underdeveloped countries respectively, where fungi like *Botrytis* spp., *Aspergillus* spp., *Mucor* spp., *Fusarium* spp., *Thielaviopsis* spp., *Verticillium* spp., *Ustilago* spp., *Rhizoctonia* spp. and *Puccinia* spp. are in the first line of implication [3,4,5]. Their virulence exhibits considerable diversity both in their developmental biology and in the types of induced symptoms [6].

Over time, several scientific practices have been used to attenuate and/or eliminate fungal pathogens from agricultural lands. The application of fungicides to control phytopathogens effects is one of the most important tools exploited for plant protection and crop improvement. Nevertheless, their regular use present a serious risk for environment, especially if residues persist in soil or migrate to waterways, hence damaging soil and water quality [4]. In addition, resistance emergence in phytopathogen populations to these chemicals, their low ability to penetrate in plant tissues, their high cost application, and the deleterious effects caused by fungicides to some beneficial organisms in soil constitute difficult obstacles [7-9].

Under pressure of the aforementioned problems, scientific and farmers were obliged to find other alternatives to reduce fungicides utilization such as selection, cultivation

and breeding of resistant crops [7]. In the last few years, a new ecofriendly and cost-effective approach consisting to use beneficial soil bacteria to control plant pathogens, but also to improve plant growth under different conditions, was widely adopted by both scientists and farmers. This kind of telluric microorganism is referred to as Plant Growth Promoting Bacteria or rhizobacteria (PGPBR) [10]. PGPR can affect plant growth by providing elementary elements essential for plant growth, producing secondary metabolites like phytohormones, antibiotics, biologically active compounds etc., and suppressing or decreasing phytopathogens via competition [11].

Beside their antagonism, some PGPR can induce biochemical and physiological modifications in plant tissues, improving their defenses against pathogens, which is designated by the term Induced Systemic Resistance (ISR) [12]. Several works proved that bacteria belonging to the genera *Acinetobacter*, *Pseudomonas*, *Staphylococcus*, *Bacillus*, *Enterobacter*, *Burkholderia* and *Alcaligenes* are known to be PGPR capable to exhibit high antagonistic activities against phytopathogenic fungi like *Fusarium solani*, *Pythium ultimum*, *Alternaria alternata*, *Botrytis cinerea* and *Phytophthora cryptogea* [13, 14, 15 and 16].

In this work, the bacterial isolate (RhiNA) was selected, among others, based on multi-PGP-traits and antifungal metabolites screening. It was then molecularly identified and tested for its ability to inhibit mycelial growth and spores germination of four fungi *Botrytis cinerea*, *Aspergillus flavus*, *A. niger* and *Mucor* sp. Finally, this strain was assessed *in vivo* to demonstrate the antagonistic efficiency against *Botrytis cinerea*-BC1 affecting apple fruits.

## II. METHODS AND MATERIAL

### 1. Bacterial strain and Plant pathogens

The strain RhiNA was isolated in 2014 by the Laboratory of Mastery of Renewable Energies from an agricultural soil in northern Algeria (Bejaia). A reference strain Pseudomonas protegens $CHAO^T$ was kindly provided by Professor Anton Hartmann from the German Research Center for Environmental Health (GmbH), Research Unit Microbe-Plant Interactions-Germany. The phytopathogen fungus Botrytiscinerea-BC1 was generously provided by the laboratory of mycology-University A. Mira of Bejaia-Algeria. This strain was originally isolated in France by the Laboratory of Plant Protection (INRA-STPV, Avignon).The fungi Aspergillus niger, A. flavus and Mucor sp. were offered by the laboratory of applied microbiology, University of Bejaia-Algeria. Apple fruits (Malus domestica) variety Golden Delicious were freshly harvested in Bejaia-Algeria, and immediately transported to the laboratory before use.

### 2. Production of Antifungal Compounds

#### A. Cyanhydric acid (HCN)

HCN detection was carried out following Lorck's protocol (1948) [17]. For this, a nutrient agar was supplemented with filter-sterilized glycine solution to a final concentration of 4.4 g/L. The medium was then casted in Petri dishes (9 mm of diameter). The covers were upholstered by Whatman paper impregnated with a sodium picrate solution (5% picric addition of 2% of anhydrous sodium carbonate acid). 100 uL of bacterial culture was flooded on agar using sterile swabs. Petri dishes were sealed with parafilm and incubated at 30°C/96 h. Ammonia production is expressed by transformation of the yellow color on the Whatman paper (provided by the solution of sodium picrate) to orange or brown.

#### B. Ammonia

For ammonia detection, 100 µL of fresh culture (DO = 0.7) was inoculated in tubes containing 10 mL of peptone broth medium. After incubation (30°C/96 h), 500 µL of Nessler reagent were added to each tube. A positive result is designated by apparition of yellow or orange color in the mixture, indicating ammonia production by strain [18].

#### C. Chitinase assay

Chitinase was screened according to the protocol of [19]. Agar medium with the following composition in 1 L of distilled water ; K2HPO4 (2.7 g) ; KH2PO4 (0.3 g) ; MgSO47H2O (0.7g ) ; NaCl (0.5 g) ; KCl (0.5 g) ; yeast extract (0.13 g) ; Agar (15) ; 7.2 ± 0.02 pH was supplemented with colloidal chitin (0.8 à 0.6 g). This last is prepared by suspending 1 g of chitin in 9 mL of concentrated HCl. The solution is left under stirring for

2 h before bringing the volume to 250 mL. After 24 h, the obtained pellet is re-suspended in distilled water, washed 3 times with normal water and three times with distilled water then left for decantation. The mixture was filtered through metallic sieve (0.5 mm) and the filtrate was stocked at 4°C before using. The strain is inoculated by the disc agar method. A positive result is expressed by a clear hallo around the disc.

**D. Siderophores**

Siderophores were screened on Chrome azurol S (CAS). The CAS medium contains four separately prepared solutions. The Fe-CAS indicator solution (1) contained 10 mL of 1 mM $FeCl_3$-$6H_2O$ (in 10 mM HC1) and 50 mL of CAS solution (1.21 mg $mL^{-1}$); the mixture was added slowly to 40 mL of an aqueous solution of HDTMA (1.82 mg/mL). Buffer solution (2) was prepared by dissolving 30.24 g of PIPES in 750mL of a salt solution (0.3 g $KH_2PO_4$, 0.5 g NaCI, 1.0 g $NH_4C1$, 15 g agar); pH was adjusted to 6.8 using 50% KOH and water was added to bring the volume to 800 mL. The solution (3) contained per 70 mL water: 2 g glucose, 2 g mannitol, 493 mg $MgSO_4$-$7H_2O$, 11 mg $CaCI_2$, 1.17 mg $MnSO_4$-$H_2O$, 1.4 mg $H_3BO_3$, 0.04 mg $CuSO_4$-5 $H_20$, 1.2 mg $ZnSO_4$-$7H_2O$, and 1.0 mg $Na_2MoO_4$-$2H_2O$ in 70 mL of distilled water. Solutions (1) (2) (3) were autoclaved separately then cooled to 50°C. 30 mL of filter-sterilized solution (4) containing 10% (w/v) casamino acids was mixed with the solutions (3) and (2). The indicator solution was added last [20].

**3. Characterization of PGP-traits**

**A. Enzymatic activities**

To study the strain's ability to degrade various organic substrates, the agar disk method was used. Cellulolytic and esterasic/lipolytic activities were detected using the protocols proposed by [21] and [22], respectively. Proteolytic, amylolytic and ureasic activities were screened by the methods described in [23], [24] and [25], respectively.

**B. Indole 3-Acetic Acid (IAA) quantification**

IAA quantification was performed on LB medium supplemented with tryptophan [1g/L]. After incubation, 1.5 of culture was transferred to microtubes, centrifuged at 9,500xg for 2 min. 1 mL of supernatant is transferred to tubes together with 1 mL of Salkowski's reagent (1 mL of 0.5 mol $L^{-1}$ FeCl3 and 49 mL of 35 % HClO4). Half an hour later, the amount of IAA is determined by reading the mixture absorbance at 350 nm. Results are compared to those obtained with a standard curve prepared by a pure IAA (Sigma Aldrich ®). Visually, a pink color in the mixture (supernatant/Salkowski's reagent) indicates auxin's production by the stain [26].

**C. Phosphate solubilization**

The strain's ability to solubilize inorganic tricalcium phosphate was studied using Pikovskaya's medium (PKV) containing per litter: glucose, 10 g; $Ca_3$ $(PO_4)_2$, 5 g; $(NH_4)$ $2SO_4$, 0.5 g; NaCl, 0.2 g; $MgSO_4$ $7H_2O$, 0.1 g; KCl, 0.2 g; yeast extract, 0.5 g; $MnSO_4$ H2O, 0.002 g; and $FeSO_4$ $7H_2O$, 0.002 g; agar 15 g. The capacity of an isolate to solubilize inorganic phosphate is expressed by a clear halo around the agar disk [27].

All PGP-traits, except IAA and HCN, were simultaneously screamed and compared to those of reference strain Pseudomonas protegens-$CHAO^T$.

**4. Molecular identification**

Molecular identification was carried out by sequencing DNA fragments coding for the 16S rRNA. After DNA extraction, amplification was realized using universal primers (5'-S-D-Bact-0008-a-S-20-3') and (5'-S-D-Bact-1495-a-S-20-3') having the following sequences (5'-AGAGTTTGATCCTGGCTCAG-3') and (5'-AAGGAGGTGATCCAGCCGCA-3') [28]. The reactional mixture for amplification contained: 2.5µL PCR reaction buffer, 2.5 µL $MgCl_2$, 0.2µL deoxynucleoside triphosphate, 0.3µL of each primer, 0.2µL Taq DNA polymerase and 1µL of total DNA. PCR program was started at 94°C for 3 minutes, then 35 cycles of denaturation at 94°C for 45 seconds, followed annealing for 1 minutes at 55°C and elongation for 2 min at 72°C, and finished by an elongation step at 72°C for 8 minutes. The amplification product was revealed by electrophoresis (1.5% agarose gel in 0.5x Tris–borate–EDTA buffer/30 minutes). The migration product was revealed under UV detector after putting them in a 0.5 mg/l ethidium bromide solution/30min.

An enzymatic sequencing of the amplified DNA fragments was realized according to the method of Sanger. Sequences were deposited in the GenBank and compared with sequences available at the NCBI database (http://www.ncbi.nlm.nih.gov) using BLAST algorithm [29]. A phylogenetic dendrogram was constructed via the neighbor joining method and tree topology was evaluated by performing bootstrap analysis of data sets using MEGA6.

### 5. Mycelial growth inhibition

The antagonistic activity P. protegens-RhiNA against the following fungi: B. cinerea, Mucor sp., A. niger and A. flavus was studied using agar diffusion method [30]. Agar discs of fresh fungal cultures (0.5 cm diameter) were putted in the center of Petri dishes containing Potato Dextrose Agar medium (PDA). Similar discs of antagonistic bacteria are placed at 2.5 cm of the fungal disc (3 discs per plate). Plates without bacteria were used as control. After incubation (25±2° C, 5 days) the percentage of fungal growth inhibition was calculated using the formula: $[(KR-R_1)/KR] \times 100$. KR corresponds to the distance from inoculation point to fungal colony margin in the control (mm). $R_1$ represents the distance between fungal colony center and its periphery in the treated plates.

### 6. Inhibition of fungal spores' germination

In Eppendorfs tubes, 20µl of sporale suspension ($10^6$ Spores/mL) were mixed together with fresh bacterial culture of the antagonist ($10^8$ CFU/ml). 1 mL of sterile distilled water with 5% of glucose was added to each Eppendorf. The mixture is incubated at 21°C/24 h. Controls were inoculated only with fungal spores. The experiment was realized in triplicate. Spore germination percentage (SGP %) was hemocytometrically calculated using an optic microscope (40X) [31].

### 7. In vivo assay on apple fruits

In vivo test on apple fruits was monitored using a modified protocol of [32]. Fresh apple fruits with similar calibers were disinfected with sterile distilled water, supplemented with 2% hypochlorite, and left at room temperature for drying. Three equidistance holes (4 mm x 5 mm) were made in equatorial zone of fruits and each hole was inoculated with 30 µL of Bacterial suspension of the antagonist ($10^8$ CFU/mL). After 2 h, each hole was supplemented with 15 µL of B. cinerea-sporale suspension ($10^6$ Spore/mL). Apple fruits were placed in sterile boxes walled with absorbent paper humidified with sterile distilled water (3 fruits/box), then incubated at 25 ± 2°C for 4 days. At the end of the experiment, the rotten surface was measured using the ImageJ® program. The experiment was repeated in triplicate. Controls without bacterium were used.

### 8. Statistical analysis

Statistical analysis was carried using the software GraphPad Prism® version 6. The one-way ANOVA test was used to compare in vitro antagonistic assay's results. In vivo assay was analyzed using impaired t test with Welch's correction ($p<0.001$).

## III. RESULTS AND DISCUSSION

### 1. PGP-traits

The strain RhiNA was able to produce high amounts of HCN (Whatman paper color has changed from yellow to brown in the three inoculated replicates). In presence of tryptophan (1 mg/mL), this strain produced 68.67±4.84 µg/mL of the auxin-IAA. The reference strain (P. protegens $CHAO^T$) produced also different studied enzymes except chitinase, while the strain RhiNA showed high level of protease, lipase, esterase, urease and moderate amounts of chitinase. The tow strains produced considerable amount of siderophores on CAS-agar medium and to solubilized tricalcium phosphate [$Ca_3(PO_4)_2$] on Pikovskaya's medium (Table 1).

**Table 1:** PGP-traits of the isolate RhiNA and *Pseudomonas protegens* $CHA0^T$

| PGP-traits | RhiNA | CHAO$^T$ | PGP-traits | RhiNA | CHAO$^T$ |
|---|---|---|---|---|---|
| IAA (µg/mL) | 6.86±0.48 | / | Esterase | ++ | + |
| HCN | +++ | ++ | Cellulase | +++ | + |
| Siderophores | +++ | ++ | Lipase | ++ | ++ |
| Chitinase | + | - | Urease | - | + |
| Protease | ++ | ++ | (P) solubilization | ++ | ++ |

Zones of clearance or indicator color changes around colonies were measured; (+): diameter between 7 and 10 mm; (++): diameter between 10 and 20 mm; (+++): diameter above 20 mm; (-): negative result.

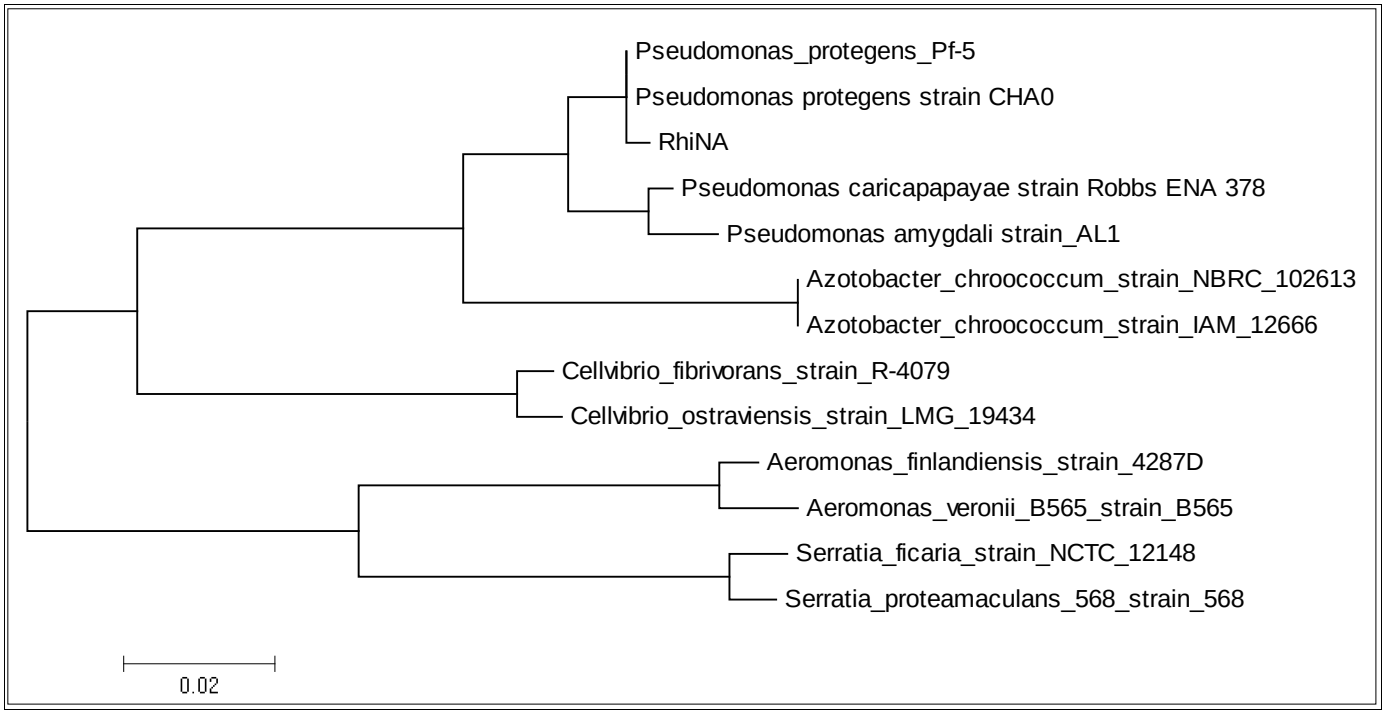


**Figure 1:** Neighbor-Joining Tree obtained using MEGA6, revealing the phylogenetic relationship of the analyzed isolates. -Bar indicates 2% sequence divergence.

## 2. Mycelial growth inhibition

The results showed that the strain RhiNA exerted potential inhibition of mycelial growth when confronted to the fungi *B. cinerea*, *A. niger*, *Mucor* sp. and *A. flavus*. A highest inhibition was obtained against *A. niger* 66%±0.88, followed by *Mucor* sp. (66%±1.33). The PGI obtained against both *B. cinerea* and *A. flavus* were 62±1.33 and 58 %±1.33, respectively. No significant differences were noted between the different PGI against the four fungi (Figure 2).

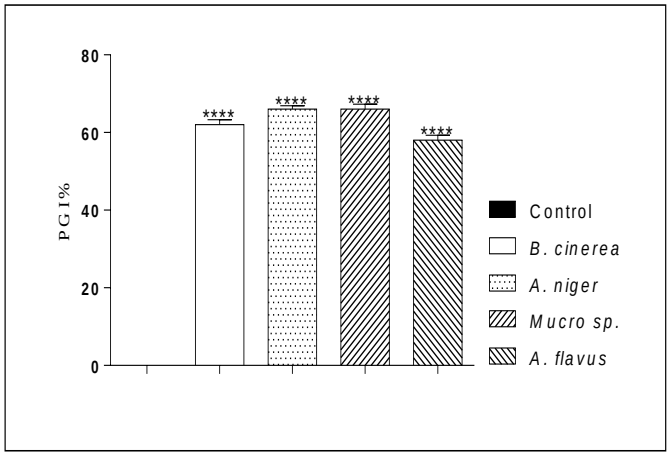


**Figure 2:** Percentage of mycelial growth inhibition (PGI %) of *B. cinerea*, *A. niger*, *Mucor* sp. and *A. flavus* by the strain RhiNA. ****: significant difference ($p < 0.0001$).

## 3. Inhibition of fungal spores' germination

Spore germination of all fungi was highly inhibited by the strain RhiNA. The highest inhibition was obtained

against *A. flavus* with a germination success of 10.15%±9.4, followed by *A. niger*, where 14.32%±8.38 of spores were germinated in the presence of RhiNA. Only 28.92%±2.72 of *B. cinerea* spores succeeded to germinate in presence of the antagonist. *Mucor* sp. spores were less sensitive to the presence of the strain RhiNA, with a final germination percentage of 55.10%±17.96 (Figure 3).

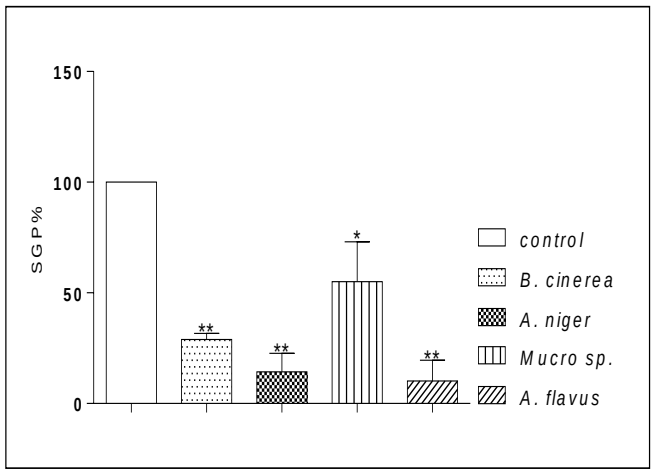


**Figure 3:** Spore germination percentage (SGP %) of B. *cinerea*, *A. niger*, *Mucor* sp. and *A. flavus* spores in presence of the strain RhiNA. *: significant difference ($p<0.05$); **: significant difference ($p<0.005$).

**4. In vivo assay on apple fruits**

In this experiment, apple fruits inoculated with *Botrytis cinerea*-BC1 showed characteristic symptoms usually generated by this fungus (gray mold). The strain RhiNA exhibited high antagonistic activity against *B. cinerea*-BC1 on apple fruits compared to the control (only inoculated with sporale suspension of *B. cinerea*). Thereby, an average area of 529.74 $mm^2$ was hit by the fungus propagation in absence of the strain RhiNA, whereas only 172.823 $mm^2$of the area was affected in presence of the strain. These proportions correspond to an inhibition percentage of 32.62% (Figures 4 and 5).

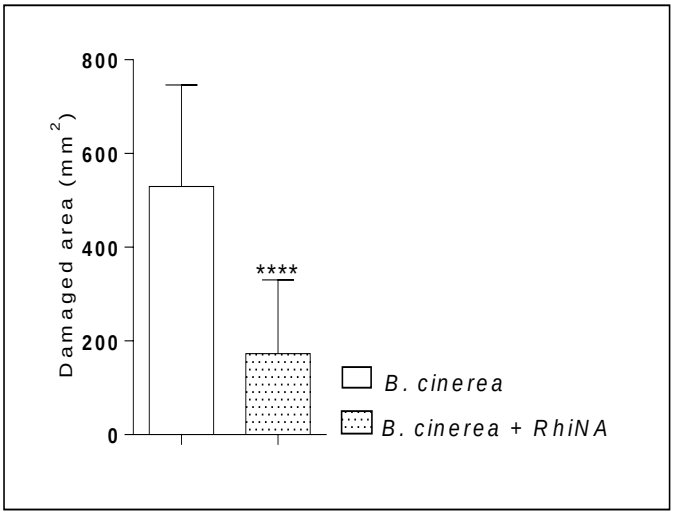


**Figure 4:** Rotten area on apple fruits attacked by *B. cinerea* in presence and absence of the antagonistic strain RhiNA, ****: significant difference ($p<0.0001$).

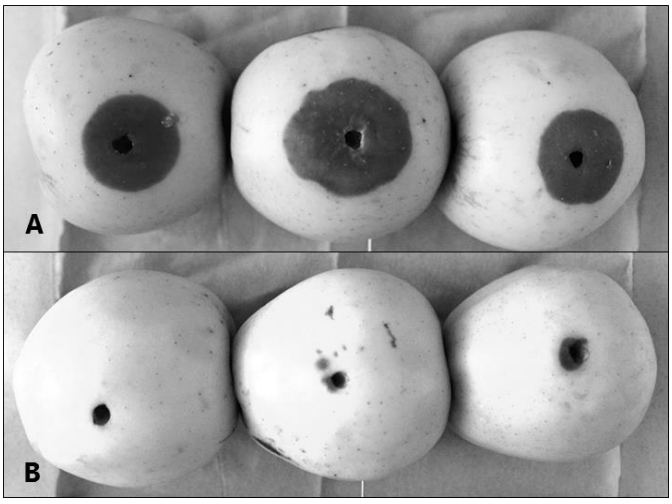


**Figure 5:** *Botrytis cinerea* gray mold on apple fruits, treated (B), or not (A), with the bacterial antagonist RhiNA after 3 days of incubation.

## IV. DISCUSSION

After identification, the strain RhiNA was affiliated to the genus *Pseudomonas*. This group of bacteria is widely distributed in nature. It plays an important role in soil biological and physicochemical processes [33]. Over than 99% of sequences similarity was established between RNA 16S of RhiNA and *Pseudomonas protegens* strain CHAO. *P. protegens* species is included in the *P. fluorescens* group, described as PGPR due to its ability to enhance plants growth and to perform vegetable crops protection against phytopathogens [34]. In addition, it is well known that *P.*

*protegens* CHAO produces secondary metabolites with large-spectrum of antibiotic activity such as HCN, $NH_3$, 2,4-diacetylphloroglucinol, pyoluteorin and pyrrolnitrin, hence exerting high phytopathogen inhibition [35]. HCN and $NH_3$ are considered as toxic metabolite and efficient inhibitors of fungal growth and development [36 and 37]. Many records signaled the implication of HCN and ammonia-producing *Pseudomonas*, including species *P. protegens*, in the biocontrol of a wide range of fungal diseases in plants [38, 39, 40, 41 and 42].

Soil organic matter (SOM) degradation, via enzymatic activities, is one of the most important processes provided by microorganisms to enhance both soil quality and plant growth. Microbial enzymes such as urease, esterase, lipase, protease, chitinase, amylase and cellulase play a crucial role in soil biological and physicochemical transformations [43, 44, 45 and 46]. In addition, enzymes such as chitinase and cellulase play an important role as biocontrol agents by degrading fungal cell walls [47]. [48] and [49] showed that several *Pseudomonas* strains produce protease, cellulase, chitinase and lipase that are implicated in SOM degradation and plant pathogens biocontrol. Another *P. protegens* (R), isolated from *Solanaceae* rhizosphere was found to be effective against broad-spectrum of phytopathogens. Its antagonistic activity was attributed to expression of to chitinase, HCN, siderophores, $NH_3$ and other secondary metabolites [50]. Otherwise, [51] underlined the role of chitinase-producing *Pseudomonas* spp. in the biocontrol of Sugarcane Red Rot Caused by *Colletotrichum falcatum* Went. [31] and [42] highlighted the role of multi-PGP-traits *P. protegens* (LiBe S5) in enhancing barely growth under heavy metal contaminated conditions, and protecting lettuce leaves against *B. cinerea*. This strain was found to produce high amounts of hydrolytic enzymes (cellulase, chitinase, amylase, urease, protease and lipase), IAA, HCN, ammonia, siderophores and phosphatase. Such characters could be implicated in the high antagonistic activity expressed by the strain RhiNA against the tested phytopathogens in this work.

Siderophores are iron-chelating compounds whose biosynthesis is regulated by iron availability in the surrounding environment. In soil, they play a key role in plant growth enhancement by improving roots iron-uptake, but also by their competitive and antagonistic characters against fungal pathogens [52, 53 and 10]. A *Pseudomonas fluorescens* WCS374r is implicated in systemic resistance induction in rice against *Magnaporthe oryzae*. This ISR is based on siderophore-mediated priming for a salicylic acid-repressible complex defense response [54]. Another siderophore-iron complex from *P. protegens* strains was found to enhance phenol content and phenol-oxidizing enzymes content in rice, inducing plant systemic resistance against the incitation of *Pyricularia oryzae* [55]. Impressively, siderophores can stimulate biosynthesis of other antimicrobial compounds implicated in plant defenses and health [56, 10].

Regarding indole acetic acid (IAA) is the most commonly produced phytohormone that interferes with different aspects of plant growth and development. Its effect on vegetables depends on plant sensitivity and the amount of IAA produced by bacteria [57]. It is well established that bacteria belonging to the genus *Pseudomonas* are excellent auxins-producers that enhance crop yield of different plants [58, 59, 60 and 26]. Moreover, some studies admit that bacterial auxins, such as IAA, mediate bacterial antagonism and stimulate plant defenses and disease suppression [61, 62]. In addition, [63] studied the role of IAA produced by *P. fluorescens* in mediating the biocontrol of *Fusarium* head blight disease of barley.

It is well known that soil phosphorus is in the second line of factors limiting plant nutrition after nitrogen. Phosphate-solubilizing microorganisms (PSM) are the most option that provides phosphorus to plants and allow avoiding fertilizers accumulation in soil [64, 65, 66, 67, 68 and 69]. [70], [71], [72], [73] and [42] highlighted the phosphate solubilization capacity of bacteria belonging to the genus *Pseudomonas* (*P. protegens*, *P. putida*, *P. fluorescens*, *P. lutea* etc.) and their implication in plant growth enhancement and crop improvement.

The bacterial antagonist RhiNA showed high inhibition of both spore germination and mycelial expansion against *B. cinerea*, *A. niger*, *A. flavus* and *Mucor sp*. The four fungi are known to be potential mycotoxins producers and plant parasites [74, 75, 76 and 77, 4]. Many researchers have reported the usefulness of bacteria belonging to the genus *Pseudomonas* in the biocontrol of these phytopathogens [78, 79 and 80]. *B.*

*cinerea* is responsible of considerable pre- and postharvest loses in many crops worldwide [81]. In our study, the application of this fungus on apple leaded to characteristic gray mold expansion from the inoculation point. During storage, the contamination between adjacent fruits may frequently acquire leading to considerable loses [32]. [82] Found that a dual culture of *Pseudomonas fluorescens* P-5 and *Bacillus subtilis* B-3 reduced *B. cinerea*-gray mold in apple fruits to 60% after 20 day of their application. They also proved the absence of significant difference between these antagonists and thiabendazol effects against *B. cinerea* affecting apple fruits. In addition, another *Pseudomonas cepacia* was used by [83] to control *B. cinerea*-gray mold in apple fruits. Application of this strain saved 20 % of the damaged area compared to control.

## V. CONCLUSION

The isolate *P. protegens* RhiNA is a multi-PGP traits bacterium that displayed high ability to inhibit mycelial growth and spores germination of *B. cinerea*, *A. niger*, *A. flavus* and *Mucor* sp. It also decreased gray mold caused by *B. cinerea* on apple fruits. Thus, this strain could be valorized and efficiently used as a biocontrol agent in agriculture.

## VI. REFERENCES


[1] Lafe, O. 2013. Agriculture, in: Lafe, O. (Ed.), Abulecentrism, Rapid Development of Society Catalyzed at the Local Community Level. Springer International Publishing., Switzerland, pp, 127-132.

[2] Singh, P.K., Kumar, V., Maurya, N., Choudhary, H., Kumar, V., Gupta, A.K. 2014. Grassroots Solutions to Overcome Abiotic and Biotic Environmental Stress in Agriculture, in: Singh, S.B., et al. (Eds.), Translational Research in Environmental and Occupational Stress. Springer., India, pp, 11-16.

[3] Glick, B.R. 2015. Biocontrol Mechanisms, in: Glick, B.R. (Ed.), Beneficial Plant-Bacterial Interactions. Springer International Publishing., Switzerland, pp. 123-157.

[4] Peng, X.D., Huang, S.L., Lin, S.H. 2015. First report of corn kernel brown spot disease caused by Mucor irregularis in China. J. Plant Dis. 99(1), 159-160.

[5] Reddy, P.P. 2015. Impacts on Plant Pathogens, in: Reddy, P.P. (Ed.), Climate Resilient Agriculture for Ensuring Food Security. Springer., India, pp, 151-177.

[6] Soanes, D.M., Skinner, W., Keon, J., Hargreaves, J., Talbot, N.J. 2002. Genomics of Phytopathogenic Fungi and the Development of Bioinformatic Resources. MPMI. 15(5), 421-427.

[7] Paoletti, M.G., Pimentel, D. 1996. Genetic Engineering in Agriculture and the Environment, Assessing risks and benefits. BioScience. 46(9), 665-673.

[8] Komárek, M., Čadková, E., Chrastný, V., Bordas, F., Bollinger, J.C. 2010. Contamination of vineyard soils with fungicides: A review of environmental and toxicological aspects. Environ. Int. 36, 138-151.

[9] Hollomon, D.W. 2015. Fungicide Resistance: 40 Years on and Still a Major Problem, in: Ishii, H. Hollomon, D.W. (Eds.), Fungicide Resistance in Plant Pathogens. Springer., Japan, pp. 3-11.

[10] Beneduzi, A., Ambrosini, A., Passaglia, L.M.P. 2012. Plant growth-promoting rhizobacteria (PGPR): Their potential as antagonists and biocontrol agents. Genet. Mol. Biol. 35 (4), 1044-1051.

[11] Vejan, P., Abdullah, R., Khadiran, T., Ismail, S and Boyce, A.N. 2016. Role of Plant Growth Promoting Rhizobacteria in Agricultural Sustainability-A Review. Molecules. doi:10.3390/molecules21050573.

[12] Choudhary, D.K., Prakash, A., Johri, B.N. 2007. Induced systemic resistance (ISR) in plants: mechanism of action. Indian J. Microbiol.47, 289-297.

[13] Annapurna, K., Kumar, A., Kumar, L.V., Govindasamy, V., Bose, P., Ramadoss, D. 2013. PGPR-Induced Systemic Resistance (ISR) in Plant Disease Management, in: Maheshwari, D.K. (Ed.), Bacteria in Agrobiology: Disease Management. Springer-Verlag Berlin, Heidelberg, pp. 405-425.

[14] Reddy, P.P. 2013. Plant Growth-Promoting Rhizobacteria (PGPR), in: Reddy, P.P. (Ed.), Recent Advances in Crop Protection. Springer., India, pp, 131-158.

[15] Gupta, G., Parihar, S.S., Ahirwar, N.K., Snehi, S.K., Singh, V. 2015. Plant Growth Promoting Rhizobacteria (PGPR): Current and Future Prospects for Development of Sustainable Agriculture. J. Microb. Biochem. Technol. 7(2), 96-102.

[16] Nadeem, S.M., Naveed, M., Ahmad, M., Zahir, Z.A. 2015. Rhizosphere Bacteria for Crop Production and Improvement of Stress Tolerance: Mechanisms of Action, Applications, and Future Prospects, in:

Arora, N.K. (Ed.), Plant Microbes Symbiosis: Applied Facets. Springer., India, pp, 1-36.
[17] Lorck, H. 1948. Production of hydrocyanic acid by bacteria. Physiol. Plant. 1, 142-146.
[18] Cappucino, J.C. Sherman, N., 1992. Negative staining. Microbiology: A laboratory Manual. 3, 125-179.
[19] Kopečný, J., Hodrová, B., Stewart, C.S. 1996. the isolation and characterization of a rumen chitinolytic bacterium. Lett. Appl. Microbiol. 23, 195-198.
[20] Alexander, B., Zuberer, D.A. 1991. Use of chrome azurol S reagents to evaluate siderophore production by rhizosphere bacteria. Biol. Fertil. Soils. 12, 39-45.
[21] Carder, J.H. 1986. Detection and quantification of cellulase by congo red staining of substrates in a cup-plate diffusion assay. Anal. Biochem. 153, 75-79.
[22] Sierra, G.A. 1957. A simple method for the detection of lipolytic activity of microorganisms and some observations on the influence of the contact between cells and fatty substrates. A. Van. Leeuw. 28, 15-22.
[23] Kavitha, T., Nelson, R., Jesi, S.J. 2013. Screening of rhizobacteria for plant growth promoting traits and antifungal activity against charcoal rot pathogen macrophomina phaseolina. Int. J. Pharm. Bio. Sci. 4, 177-186.
[24] Vinoth, R.S., Kanikkai, R.A., Babu, V.A., Manoj, G.T., Naman, H.S., Johnson, A.J., Infant, S.B., Sathiyaseelan, K. 2009. Study of starch degrading bacteria from kitchen waste soil in the production of amylase by using paddy straw. Recent. Res. Sci. Technol. 1(1), 008–013.
[25] Christensen, W.B. 1946. Urea Decomposition as a Means of Differentiating Proteus and Paracolon Cultures from Each Other and from Salmonella and Shigella Types. J. Bacteriol. 52(4), 461–466.
[26] Malik, D.K., Sindhu, S.S. 2011. Production of indole acetic acid by Pseudomonas sp.: effect of coinoculation with Mesorhizobium sp. Cicer on nodulation and plant growth of chickpea (Cicer arietinum). Physiol. Mol. Biol. Plants. 17(1), 25-32.
[27] Sagervanshi, A., Kumari, P., Nagee, A., Kumar, A. 2012. Isolation and characterization of phosphate solubilizing bacteria from an agriculture soil. Int. J. Life Sci. Pharma. Res. 2, 256-266.
[28] Daffonchio, D., Cherif, A. Borin, S. 2000. Homoduplex and Heteroduplex Polymorphisms of the Amplified Ribosomal 16S-23S Internal Transcribed Spacers Describe Genetic Relationships in the "Bacillus cereus Group". Appl. Environ. Microbiol. 66(12), 5460–5468.
[29] Kumar, S., Tamura, K. and Nei, M. 2004. MEGA3: integrated software for Molecular Evolutionary Genetics Analysis and sequence alignment. Brief. Bioinform. 5(2), 150-163.
[30] Sagahón, I.P., Anducho-Reyes, M.A., Silva-Rojas, H.V., Arana-Cuenca, A., Tellez-Jurado, A.A., Cárdenas-Álvarez, I.O., Mercado-Flores, Y. 2011. Isolation of Bacteria with Antifungal Activity against the Phytopathogenic Fungi Stenocarpella maydis and Stenocarpella macrospora. Int. J. Mol. Sci. 12, 5522-5537.
[31] Bensidhoum, L., RAI, A., Tabli, N., Nabila, K., Meriem, K., Nabti, E. 2015. Biological Control of Botrytis cinerea by Bacillus sp. Strain S7LiBe under Abiotic Stress. First International Symposium of Applied Biology CIBA USTO-MB. IJSRST. 1, 7-14.
[32] Xiao, C.L., Kim, Y.K. 2008. Postharvest fruit rots in apples caused by Botrytis cinerea, Phacidiopycnis washingtonensis, and Sphaeropsis pyriputrescens. Online. Plant Health Progress. doi:10.1094/PHP-2008-0919-01-DG.
[33] Kahlon, R.S. 2016. Pseudomonas: Genome and Comparative Genomics, in: Kahlon, R.S. (Ed.), Pseudomonas: Molecular and Applied Biology. Springer International Publishing., Switzerland, pp. 127-191.
[34] Garrido-Sanz, D., Meier-Kolthoff, J.P., Göker, M., Martín, M., Rivilla, R., Redondo-Nieto, M. 2016. Genomic and Genetic Diversity within the Pseudomonas fluorescens Complex. PLoS ONE. doi:10.1371/journal.pone.0150183.
[35] Jousset, A., Schuldes, J., Keel, C., Maurhofer, M., Daniel, R., Scheu, S., Thuermer, A. 2014. Full-genome sequence of the plant growth-promoting bacterium Pseudomonas protegens CHA0. Genome. Announc. doi:10.1128/genomeA.00322-14.
[36] McCallan, S.E.A., Weedon, F.R. 1940. Toxicity of ammonia, chlorine, hydrogen cyanide, hydrogen sulfide, and sulfide dioxide gazes. II fungi and bacteria. Contrib. Boyce. Thompson Inst. 11, 331-342.
[37] Howell, C.R., Beier, R.C., Stipanovic, R.D. 1988. Production of ammonia by Enterobacter cloacae and its possible role in the biological control Pythium preemergens Damping-off by the bacterium. Phytopathology. 78(8), 1075-1078.
[38] 5Sétamou, M., Cardwell, K.F., Schulthess, F., Hell, K. 1997. Aspergillus flavus Infection and Aflatoxin Contamination of Preharvest Maize in Benin. Plant Disease. 81(11), 1323-1327.

[39] O'Sullivan, D.J., O'Gara, F. 1992. Traits of Fluorescent Pseudomonas spp. Involved in Suppression of Plant Root Pathogens. Microbiological Reviews. 56(4), 662-676.

[40] Dowling, D.N. O'Gara, F. 1994. Metabolites of Pseudomonas involved in the biocontrol of plant disease. TIBTECH. 12, 133-141.

[41] Pal, K.K., Tilak, K.V.B.R., Saxena, A.K., Dey, R., Singh, C.S. 2000. Antifungal characteristics of a fluorescent Pseudomonas strain involved in the biological control of Rhizoctonia solani. Microbiol. Res. 155, 233-242.

[42] Walsh, U.F., Morrissey, J.P., O'Gara, F. 2001. Pseudomonas for biocontrol of phytopathogens: from functional genomics to commercial exploitation. Curr. Opinion Biotechnol. 12, 289-295.

[43] Bensidhoum, L., Nabti, E., Tabli, N., Kupferschmied, P., Weiss, A., Rothballer, M., Schmid, M., Keel, C., Hartmann, A. 2016. Heavy metal tolerant Pseudomonas protegens isolates from agricultural well water in northeastern Algeria with plant growth promoting, insecticidal and antifungal activities. Eur. J. Soil Biol. 75, 38-46.

[44] Glick, B.R. 1995. the enhancement of plant growth by free-living bacteria. Can. J. Microbial. 41, 109-117.

[45] Viveros, O.M., Jorquera, M.A., Crowle, D.E., Gajard, G., Mora, M.L. 2010. Mechanisms and practical considerations involved in plant growth promotion by rhizobacteria. J. Soil Sci. Plant Nutr. 10(3), 293-319.

[46] Rana, A., Saharan, B., Nain, L., Prasanna, R., Shivay, Y.S. 2012. Enhancing micronutrient uptake and yield of wheat through bacterial PGPR consortia. Soil Sci. Plant. Nutr. 58(5), 573-582.

[47] Xun, F., Xie, B., Liu, S., Guo, C. 2015. Effect of plant growth-promoting bacteria (PGPR) and arbuscular mycorrhizal fungi (AMF) inoculation on oats in saline-alkali soil contaminated by petroleum to enhance phytoremediation. Environ. Sci. Pollut. Res. 22, 598-608.

[48] Mitchell, R., Alexande, M., 1963. Lysis of soil fungi by bacteria. Can. J. Microbiol. 6, 169-177.

[49] Sindhu, S.S., Dadarwal, K.R. 2001. Chitinolytic and cellulolytic Pseudomonas sp. antagonistic to fungal pathogens enhances nodulation by Mesorhizobium sp. Cicer in chickpea. Microbiol. Res. 156, 353-358.

[50] Ahmad, N., Shinwari, Z.K., Bashir, S., Yasir, M. 2013. Function and phylogenetic characterization of rhizospheric bacteria associated with GM and non-GM maize. Pak. J. Bot. 45(5), 1781-1788.

[51] Ramyasmruthi, S., Pallavi, O., Pallavi, S., Tilak, K., Srividya, S. 2012. Chitinolytic and secondary metabolite producing Pseudomonas fluorescens isolated from Solanaceae rhizosphere effective against broad-spectrum fungal phytopathogens. Asia. J. Plant Sci. Res. 2(1), 16-24.

[52] Malathi, P., Viswanathan, R. 2013. Role of Microbial Chitinase in the Biocontrol of Sugarcane Red Rot Caused by Colletotrichum falcatum Went. EJBS. 6(1), 17-23.

[53] Simões, M., Cleto, S., Simões, L.C., Pereira, M.O. 2007. Microbial interactions in biofilms: role of siderophores and iron-dependent mechanisms as biocontrol strategies, in: Gilbert, P. et al., (Eds.), Biofilms: Coming of Age. Biofilm Club., Manchester, pp. 157-165.

[54] Hider, R.C., Kong, X. 2010. Chemistry and biology of siderophores. The Royal Society of Chemistry. 27, 637-657.

[55] De Vleesschauwer, D., Bakker, P.A.H.M., Djavaheri, M., Hofte, M. 2008. Pseudomonas fluorescens WCS374r-induced systemic resistance in rice against Magnaporthe oryzae is based on pseudobactin-mediated priming for a salicylic acid-repressible multifaceted defense response. Plant. Physiol. 148 1996-2012.

[56] Amutharaj, P., Sekar, C., Natheer, S. 2013. Development and use of different formulations of pseudomonas fluorescens siderophore for the enhancement of plant growth and induction of systemic resistance against pyricularia oryzae in lowland rice. Int. J. Pharm. Bio. Sci. 4(2), 831-838.

[57] Laslo, E., György, E., Mathé, I., Mara, G., Tamas, E., Abraham, B., Lanyi, S. 2011. Replacement of the traditional fertilizer with microbial technology: isolation and characterization of beneficial nitrogen fixing rhizobacteria. U. P. B. Sci. Bull. 73(SB), 109-114.

[58] Pant, G., Agrawal, P.K. 2014. Isolation and characterization of Indole Acetic Acid Producing Plant Growth Promoting Rhizobacteria from rhizospheric soil of Withania somnifera. JBSO. doi:10.7897/2321-6328.02687.

[59] Karnwal, A. 2009. Production of indole acetic acid by fluorescent pseudomonas in the presence of L-tryptophan and rice root exudates. J. Plant Pathol. 91(1), 61-63.

[60] Bharucha, U., Patel, K. Trivedi, U.B. 2013. Optimization of Indole Acetic Acid Production by Pseudomonas putida UB1 and its Effect as Plant

Growth-Promoting Rhizobacteria on Mustard (Brassica nigra). Agric. Res. 2(3), 215-221.
[61] Kamble, K.D., Galerao, D.K. 2015. Indole acetic acid production from Pseudomonas species isolated from rhizosphere of garden plants in Amravati. Int. J. Adv. Pharm. Biol. Chem. 4(1), 23-31.
[62] Khare, E., Arora, N.K. 2010. Effect of Indole-3-Acetic Acid (IAA) Produced by Pseudomonas aeruginosa in Suppression of Charcoal Rot Disease of Chickpea. Curr. Microbiol. 61, 64-68.
[63] Yu, T., Zheng, X.D. 2007. Indole-3-acetic acid enhances the biocontrol of Penicillium expansum and Botrytis cinerea on pear fruit by Cryptococcus laurentii. FEMS Yeast Res. 7, 459-464.
[64] Petti, C., Reiber, K., Ali, S.S., Berney, M., Doohan, F.M. 2012. Auxin as a player in the biocontrol of Fusarium head blight disease of barley and its potential as a disease control agent. BMC Plant Biology. 12(224), 1-9.
[65] Kumar, V., Aggarwal, N.K., Singh, B.P. 2000. Performance and Persistence of Phosphate Solubilizing Azotobacter chroococcum in wheat Rhizosphere. Folia. Microbiol. 45(4), 343-347.
[66] Chen, Y.P., Rekha, P.D., Arun, A.B., Shen, F.T., Lai, W.A., Young, C.C. 2006. Phosphate solubilizing bacteria from subtropical soil and their tricalcium phosphate solubilizing abilities. Appl. Soil Ecol. 34, 33–41.
[67] Hussain, M.I., Asghar, H.N., Akhtar, M.J., Arshad, M. 2013. Impact of phosphate solubilizing bacteria on growth and yield of maize. Soil Environ. 32(1), 71-78.
[68] Sharma, S.B., Sayyed, R.Z., Trivedi, M.H., Gobi, T.A. 2013. Phosphate solubilizing microbes: sustainable approach for managing phosphorus deficiency in agricultural soils. SpringerPlus. doi:10.1186/2193-1801-2-587.
[69] Khan, M.S., Zaidi, A., Ahmad, E. 2014. Mechanism of Phosphate Solubilization and Physiological Functions of Phosphate-Solubilizing Microorganisms, in: Khan, M.S. et al. (Eds.), Phosphate Solubilizing Microorganisms. Springer International Publishing., Switzerland, pp, 31-62.
[70] Nosrati, R., Owlia, P., Saderi, H., Rasooli, I., Malboobi, M.A. 2014. Phosphate solubilization characteristics of efficient nitrogen fixing soil Azotobacter strains. Iran J. Microbiol. 6(4), 285-295.
[71] Peix, A., Rivas, R.I., Regina, I.S., Mateos, P.F., Molina, E.M., Barrueco, C.R., Velázquez, E. 2004. Pseudomonas lutea sp. nov., a novel phosphate-solubilizing bacterium isolated from the rhizosphere of grasses. Int. J. Syst. Evol. Microbiol. 54, 847-850.
[72] Rosas, S.B., Andrés, J.A., Rovera, M., Correa, N.S. 2006. Phosphate-solubilizing Pseudomonas putida can influence the rhizobia–legume symbiosis. Soil Biol. Biochem. 38, 3502-3505.
[73] Park, K.H., Lee, C.Y., Son, H.J. 2009. Mechanism of insoluble phosphate solubilization by Pseudomonas fluorescens RAF15 isolated from ginseng rhizosphere and its plant growth-promoting activities. Lett. Appl. Microbiol. 49, 222-228.
[74] Sashidhar, B., Podile, A.R. 2010. Mineral phosphate solubilization by rhizosphere bacteria and scope for manipulation of the direct oxidation pathway involving glucose dehydrogenase. J. App. Microbiol. doi:10.1111/j.1365-2672.2009.04654.x.
[75] Michailides, T.J. 1991. Characterization and comparative studies of Mucor isolates from stone fruits from California and Chile. Plant Dis. 75, 373-380.
[76] Calvo, J., Calvente, V., Orellano, M.E., Benuzzi, D., Sanz, M.I. 2010. Control of Penicillium expansum and Botrytis cinerea on Apple Fruit by Mixtures of Bacteria and Yeast. Food. Bioprocess. Technol. 3, 644-650.
[77] Sharma, R. 2012. Pathogenecity of Aspergillus niger in plants. Cibtech Journal of Microbiology. 1(1), 47-51.
[78] Janisiewicz, W.J., Marchi, A. 1992. Control of storage rot on various pear cultivars with a saprophytic strain of Pseudomonas syringae. Plant. Dis. 76, 555-560.
[79] Luna-Romero, I.J., Carvajal-Moreno, M., Flores-Martínez, A., Ferrera-Cerrato, R. 2000. Possibility of Biological Control of Aspergillus flavus with Pseudomonas fluorescens on Maize Ear. Revista Mexicana de Fitopatología. 18(1), 50-54.
[80] Yunus, F.N., Iqbal, M., Jabeen, K., Kanwal, Z., Rashid, F. 2016. Antagonistic Activity of Pseudomonas fluorescens against Fungal Plant Pathogen Aspergillus niger. Sci. Lett. 4(1), 66-70.
[81] Montealegre, J.R., López, C., Stadnik, M.J., Henríquez, J.L., Herrera, R., Polanco, R., Piero, R.M.D., Pérez, L.M. 2010. Control of grey rot of apple fruits by biologically active natural products. Trop. Plant Pathol. 35(5), 271-276.
[82] Peighami-Ashnaei, S., Sharifi-Tehrani, A., Ahmadzadeh, M., Behboudi, K. 2009. Selection of bacterial antagonists for the biological control of Botrytis cinerea in apple (Malus domestica) and in

comparison with application of thiabendazole. Commun. Agric. Appl. Biol. Sci. 74(3), 739-43.

[83] Janisiewicz, W.J., Roitman, J. 1988. Biological control of blue mold and gray mold on apple and pear with Pseudomonas cepacia. Phytopathology. 78, 1697-1700.